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Rapid Communication

Analysis of Proliferative Grade Using Anti-PCNA/Cyclin Monoclonal Antibodies in Fixed, Embedded Tissues

Comparison with Flow Cytometric Analysis

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Cell kinetic information is an important adjunct to histologically-based tumor classifications. Presently, cell kinetic data can be obtained from slide-based material only with monoclonal antibodies such as Ki-67, which require the use of frozen sections and cannot be applied to archival, paraffin-embedded material. Monoclonal antibodies have recently been generated to PCNA/cyclin, a 36 kd, S-phase-associated nuclear protein. The authors investigated whether monoclonal antibody 19A2 could be used to identify proliferating cells within fixed, embedded tissue sections. Deparaffinized sections of 41 methacarn-fixed human tumors were immunostained with 19A2 using a streptavidin biotin immunoperoxidase system. A semiquantitative scoring system was used to evaluate the fraction of cells that were PCNA/cyclin-positive, and this score was compared with cell kinetic data obtained from parallel flow cytometric S-phase analysis that had been performed on fresh samples of the same tumors. While there was general agreement between the slide-based, antibody-derived and the flow cytometrically-derived cell kinetic information, some discrepancies were observed. Some of the latter represented cases in which the anti-PCNA/cyclin antibody preparations demonstrated significant heterogeneity in the numbers of proliferating cells in different regions of the tumor. In other cases, a significant fraction of the positive cells corresponded to nontumor stromal and/or inflammatory cells. In these cases, the slide-based method provided more information about the tumor cell population than did the flow cytometry data. It is concluded that semiquantitative immunocytochemical analysis with anti-PCNA/cyclin antibodies may represent a simple, reproducible, yet powerful technique for the routine analysis of cell kinetic data in alcohol-fixed, paraffin-embedded tissue by the surgical pathologist. (Am J Pathol 1989, 134:733–739)

PCNA/cyclin is a 36 kd nuclear protein, the expression of which is correlated with the S-phase of the cell cycle.[1] The protein was described independently by Bravo and Celis[2] through two-dimensional gel electrophoretic studies of proliferating and quiescent cells, and by Miyachi et al[3] through the use of human autoantibodies in lupus patients. Recently, it has been demonstrated that PCNA/cyclin is an auxiliary protein of DNA polymerase-delta[4] and plays a critical role in the initiation of cell proliferation.[5] Ogata et al[6] have recently developed murine monoclonal antibodies to PCNA/cyclin, and have used them to study the association of this protein with proliferation in human cell lines using indirect immunofluorescence and flow cytometric analyses.[7] We now demonstrate the use of one of the monoclonal antibodies, 19A2, in immunocytochemical analyses on fixed, embedded tissue sections and compare the cell kinetic information thereby derived with that obtained from flow cytometric analysis.

Materials and Methods

Tissues

A retrospective analysis of tumor tissue obtained from the Department of Pathology (Hospital Pathology) at Univer-

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sity Hospital, Seattle, between April and September 1988 was performed. As preliminary studies had indicated that the 19A2 monoclonal antibody could be used on deparaffinized alcohol-fixed but not formaldehyde-fixed tissue, the criteria for selection of tissue included the availability of paraffin blocks of methacarn-fixed tissue, as well as the presence of a report documenting the results of flow cytometric cell cycle analysis on corresponding fresh tumor. All tumors meeting these criteria were used in the study; a total of 41 such cases were available. Normal tissue used for positive and negative controls included human tonsil, small bowel, lung, endometrium, and skin. All tissues were fixed a minimum of 4 hours in methacarn fixative, processed, and embedded in paraffin.

Immunocytochemical Methods

Anti-PCNA/cyclin monoclonal antibody 19A2, a murine IgM antibody, originally developed by Ogata et al,[6] was obtained from American Biotech (Plantation, FL) as undiluted ascites fluid. After titration experiments described in the Results section, the antibody was employed in a streptavidin-biotin immunoperoxidase method, which is a modification of the avidin-biotin immunoperoxidase method described previously.[8] Briefly, after deparaffinization through graded alcohols, endogeneous peroxidase activity was blocked by incubating the slides in 0.1% hydrogen peroxide with 1% sodium azide for 10 minutes. After washing in buffer A (phosphate buffered saline [PBS] with 0.1% bovine serum albumin [BSA] and 0.01% Triton X-100), the diluted anti-PCNA/cyclin antibody was overlaid on the tissue sections for 1 hour at room temperature. After washes in buffer A, biotinylated anti-mouse IgG (which cross reacts with mouse IgM; Vector Laboratories, Burlingame, CA) was applied at a dilution of 1:1000 and incubated for 30 minutes at room temperature. After washes in buffer A, streptavidin-peroxidase (Jackson Immunonuclear Laboratories; Westgrove, PA) was applied to the sections at a 1:5000 dilution into PBS with 1% BSA for 30 minutes at room temperature. Slides were developed, following additional washes in buffer A, using 3,3′-diaminobenzidine in 0.1 M Tris buffer, pH 7.6, with the nickel chloride color enhancement method as previously described.[8,9]

Scoring of PCNA/cyclin Immunocytochemistry

At least five representative high-power fields were examined in all cases; tumors were independently and blindly graded by at least two observers using a semiquantitative scale of 1 through 4, corresponding to estimated quartiles of tumor cell nuclear immunostaining (1 = 0 to 25%; 2 = 26 to 50%; 3 = 51 to 75%; 4 = 76 to 100%). In cases where significant field-to-field variation in the percentage of positive cells existed, a major and minor score were indicated.

Flow Cytometry

Data was obtained from flow cytometry reports in patient records which had been generated from analysis on a Becton Dickenson FACS analyzer. Standard techniques of fresh tissue preparation for flow cytometric analysis had been employed.[10,11] Cell cycle parameters had been analyzed by the method of Dean and Jett.[12] The calculated S-phase data from these reports were recorded, as well as the interpretation of the proliferative rate as "low" (<6% S-phase), "intermediate" (6 to 8% S-phase), or "high" (>8% S-phase).

Results

Titration of PCNA/Cyclin Antibody on Normal Tissue

Serial dilution studies demonstrated that 1:16,000 represented the optimal dilution of the ascites fluid as tested on the positive control material. At this optimal dilution, immunostaining was restricted to the nuclei of positive cells. Proliferating cell compartments, such as the germinal centers of tonsillar lymphoid tissue and the cells within the crypts of the intestinal mucosa, were clearly identified by nuclear immunostaining, as indicated in Figure 1.

Analysis of Human Tumor Tissues

At least one block of methacarn-fixed, paraffin-embedded tissue was examined for each of the 41 tumors. PCNA/cyclin scores, representing the fraction of tumor nuclei read as positive with the anti-PCNA/cyclin antibody, ranged from 1 to 4; these results are summarized in Table 1, with examples of low and high scores of anti-PCNA/cyclin immunostaining of two different nonsmall cell lung carcinomas presented in Figure 2. Although there was some variation in the intensity of the nuclear immunostaining in different positive cells, all immunostained nuclei, independent of intensity, were scored as positive. Defining primary PCNA/cyclin scores of 1 as "low," 2 or 3 as "intermediate," and 4 as "high," correlation of the PCNA/cyclin scores with the flow cytometric analysis was generally good. A significant discrepancy between the flow cytometry-generated and immunocytochemically-deter-

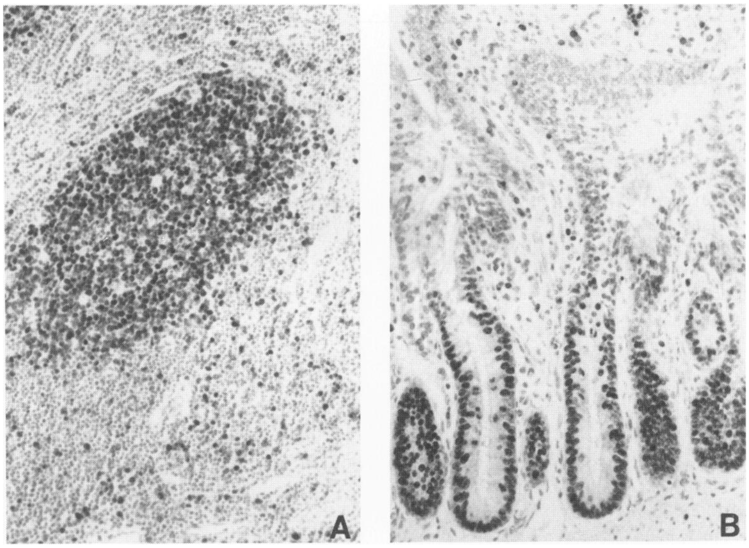

Figure 1. *Anti-PCNA/cyclin immunocytochemistry on methacarn-fixed, deparaffinized sections of human tonsil* (**A**) *and intestine* (**B**). *Note immunostaining of nuclei of cells of germinal center of tonsil and nuclei of cells at base of crypt of intestinal glands. Streptavidin-biotin immunoperoxidase method; A, original magnification × 125; B, original magnification × 160.*

mined proliferation indices was present in 6 of the 41 cases, however.

As indicated in Table 1, in four of these cases (three breast adenocarcinomas and one neurendocrine carcinoma), the anti-PCNA/cyclin immunocytochemistry score was significantly higher than the flow cytometrically-determined proliferation index, but in two sarcoma cases the reverse was true.

In two additional cases, there were insufficient cell numbers obtained by flow cytometry to ascertain reliable S-phase numbers, and in one case flow cytometry histograms had been difficult to interpret owing to relatively large numbers of necrotic cells. In all these latter cases, however, it was possible to obtain PNCA/cyclin scores by selecting microscopic fields with adequate numbers of non-necrotic tumor cells.

Discussion

We have demonstrated that it is possible to employ a commercially-available monoclonal antibody to PCNA/cyclin to extract cell kinetic data from fixed, embedded sections of human tumors. The data thereby obtained correlate well with that obtained from parallel flow cytometric analyses.

It is instructive, however, to further evaluate the cases in which there was significant discrepancy between the cell kinetic data obtained by the two methods. In several of the cases, examination of the immunostained slides revealed that there was considerable field-to-field variation in the fraction of PCNA/cyclin-positive tumor nuclei. Sampling errors in the tissue obtained for flow cytometric analysis could therefore account for the discrepancy, ie, tissue from different parts of the tumor used in the two different methods may have differed with respect to proliferative rates. An example of this intrinsic heterogeneity is demonstrated in Figure 3, a case of metastatic melanoma. Other factors may also explain differences in kinetic indices as obtained by these two methods, such as the amount of stromal cells and inflammatory cells, many of which can demonstrate significant proliferation, and the proportion of which, relative to the neoplastic cell population, can vary significantly from region to region within a tumor. In three cases, accurate S-phase assessment was not possible with flow cytometry because of the relatively small number of cells obtained and/or the presence of extensive necrosis. In these cases, the anti-PCNA/cyclin antibody technique was able to circumvent these problems by permitting visual analysis of the tissue; viable tumor areas could clearly be evaluated and separated from necrotic zones. In summary, in many cases, more information can be obtained by using a slide-based system in which the observer can simultaneously evaluate tumor cell kinetics and histology.

Different methods have been used for studying cell kinetics in tumor specimens. These include: 1) tritiated thymidine incorporation followed by autoradiography; 2) bromodeoxyuridine incorporation followed by anti-BrdU immunocytochemistry; 3) flow cytometric analysis; 4) immunocytochemistry using cell cycle specific monoclonal antibodies.

Radiolabeling with tritiated thymidine has numerous drawbacks, including: the requirement of fresh, viable tissue for thymidine incubation; problems with uniform uptake and potential alterations of cell kinetics in the tumor samples incubated *in vitro;* and the expense and dangers of handling radioactive substances. Use of BrdU and anti-BrdU antibodies eliminates some of these disadvantages, but still retains the requirement of using fresh, viable tis-

Table 1.

Tumor	Site	Proliferative grade with anti-PCNA/cyclin*	Calculated %S-phase by flow cytometry†
Sarcoma, NOS	Chest wall	1	1–4 (low)
Schwannoma	Parapharyngeal	1	5 (low)
Liposarcoma	Lung	1	2 (low)
Synovial sarcoma	Lower extremity	1 (2)	2 (low)
Adenocarcinoma	Lung	1 (2)	1 (low)
Adenoma	Thyroid	1	2 (low)
Osteogenic sarcoma	Ulna	1	2 (low)
Carcinoid tumor	Omentum	1	3 (low)
Myxoma	Lower extremity	1	– (very low‡)
Adenocarcinoma	Prostate	1	1 (low)
Sarcoma, NOS	Retroperitoneum	2	2 (low)
Melanoma, metastatic	Adrenal	2 (1) (3)	6 (intermediate)
Fibroma	Ovary	2	3 (low)
Giant cell tumor	Tibia	2 (3)	4 (low)
Sarcoma (MFH)§	Upper extremity	2 (1)	approx. 30‡ (high)
Leiomyosarcoma§	Soft tissue	2 (3)	11 (high)
Adenocarcinoma	Breast	2 (3)	7 (intermediate)
Adenocarcinoma§	Breast	3	3 (low)
Sarcoma, NOS	Upper extremity	3 (4)	23 (high)
Adenocarcinoma	Breast	3	26 (high)
Adenocarcinoma	Breast	3	24 (high)
Neurendocrine carcinoma§	Paratracheal node	3 (4)	3 (low)
Leiomyosarcoma	Thigh	3	20 (high)
Mesothelioma	Chest wall	3 (2)	12 (high)
Adenocarcinoma§	Breast	3	4 (low)
Non-Hodgkins lymphoma	Cervical node	3 (2)	6 (high)
Adenocarcinoma	Mediastinal node	3	–ǁ
Sarcoma, NOS	Chest wall	3 (2)	12 (high)
Carcinoma	Cervix	3	24 (high)
Adenocarcinoma§	Breast	3 (4) (2)	3 (low)
Adenocarcinoma	Breast	4	10 (high)
Embryonal carcinoma	Lung	4	23 (high)
Adenocarcinoma	Lung	4	10 (high)
Carcinoma	Cervix	4	6 (intermediate)
Adenocarcinoma	Breast	4 (3)	12 (high)
Adenocarcinoma, intraductal	Breast	4	27 (high)
Medullary carcinoma	Breast	4 (3)	24 (high)
Carcinoma	Cervix	4	27 (high)
Osteogenic sarcoma	Lower extremity	4 (3)	27 (high)
Sarcoma, NOS	Lower extremity	4 (3)	17 (high)
Adenocarcinoma, metastatic	Breast	4 (3)	15 (high)

* Number in parenthesis indicates secondary grade for heterogenous tumor populations (see text for details).
† See text for interpretation of proliferative index.
‡ Too few cells to accurately assess %S.
§ Cases with discrepancies between flow cytometrically-determined S-phase data and PCNA/cyclin score.
ǁ Too much necrosis present to determine %S.

sue for incubations. Flow cytometrically determined kinetic analysis has many advantages over other methods, including the ability to use fresh as well as archival, paraffin-embedded material, but flow cytometry has its own limitations, most notably the requirement for digested tissues samples, which by definition cannot be used for simultaneous histologic interpretation. When digested tissue is obtained from paraffin blocks, present extraction methods result in stripped nuclei with significant loss of cell identification data. Immunocytochemical methods have important intrinsic advantages over destructive techniques such as flow cytometry, most important of which is the preservation of tissue architecture and cell-to-cell relationships. Indeed, anti-PCNA/cyclin antibodies can be run as part of a larger panel of monoclonal antibodies in which other markers of tumor differentiation are included. If necessary, it is also possible to perform double-labeling immunocytochemical procedures with anti-PCNA/cyclin and other cell-specific monoclonal antibodies to positively identify the proliferating cells on a single tissue section. Finally, through the use of quantitative microscopy systems,[13] it should be possible to derive even more precise data from anti-PCNA/cyclin immunostained preparations.

Several monoclonal antibodies have been developed that preferentially label proliferating cells. These include Ki-67,[14] anti-PAA,[15] anti-ribonucleotide reductase,[16] anti-DNA polymerase alpha,[17] and C_5F_{10},[18] an anti-M phase-specific monoclonal antibody. These other antibodies are less useful reagents, however, because some, like Ki-67,

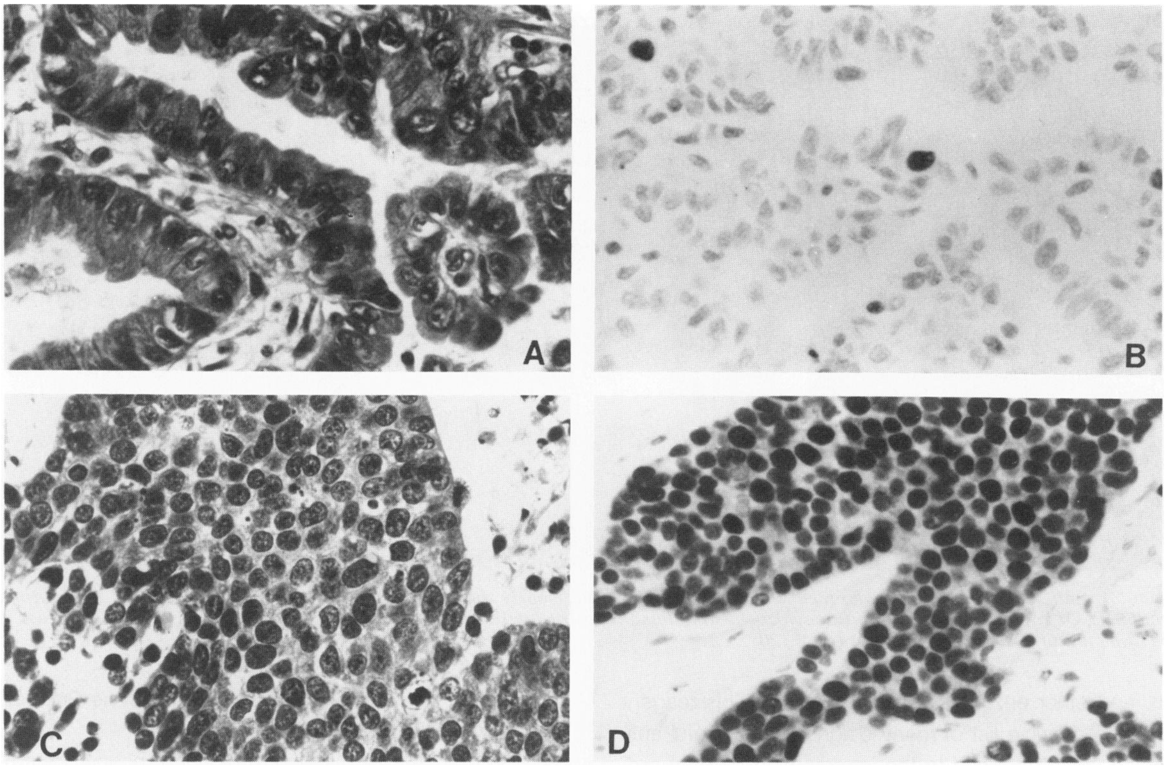

Figure 2. *Methacarn-fixed, deparaffinized sections of two different nonsmall cell lung carcinomas, manifesting low and high proliferative indices. H & E-stained section* **(A)** *of a well-differentiated carcinoma, with corresponding PCNA/cyclin immunolocalization* **(B)**, *demonstrating low proliferative grade.* **C:** *H & E-stained section of a poorly differentiated carcinoma, with corresponding PCNA/cyclin immunolocalization* **(D)** *demonstrating a tumor with a high 'proliferative grade'. Original magnifications ×320.*

can be used only on fresh, frozen sections, and, in the case of others, such as those to PAA or ribonucleotide reductase, the distribution of the corresponding antigens throughout the cell cycle and their presence in different tissue types has not been well studied. In contrast, the cell-cycle distribution of PCNA/cyclin has been well described, with peak concentrations seen in late G1/S-phases.[19,20] Additionally, the S-phase has been further subdivided on the basis of PCNA/cyclin patterns.[1]

It remains to be explained, however, why the percentages of positive tumor cells are much greater with the PCNA/cyclin antibody method than with the percentage of S-phase cells calculated from the flow cytometry histograms. If PCNA/cyclin were truly S-phase-specific, one might expect comparable numbers with both analyses; the percentage of PCNA/cyclin-positive cells, however, is consistently higher than the S-phase fractions calculated from the flow cytometry histograms. Several phenomena may account for this: 1) the S-phase numbers, as calculated by flow cytometry, include both tumor and largely nonproliferating stromal and inflammatory cell compartments, and may thus underestimate true tumor S-phase indices; 2) the anti-PCNA/cyclin antibody may be detecting persistent antigen in non-S-phase proliferating (ie, non-G_0) cells in methacarn-fixed, paraffin-embedded tissues. The presence of different immunostaining intensities in the anti-PCNA/cyclin slide preparations may reflect the latter, although stereologic and other factors may also account for variations in nuclear immunostaining intensity. For the purposes of this study, cells were read as either positive or negative, without respect to quantitation. It may well be that, through the use of quantitative microscopy,[13] additional cell cycle-related information may be forthcoming. Such studies are currently underway in this laboratory.

For this study, we chose to use a proliferative grading system based on quartiles, rather than direct quantitation of the fraction of PCNA/cyclin-positive tumor cells, to test the validity of a simple, reproducible method that could be easily exploited by the diagnostic pathologist. More rigorous counting methods might yield even more information that might be masked by this semiquantitative method, but it is unlikely that laborious cell counting techniques would be used routinely in clinical practice. This may change, however, with the recent availability of personal computer-based quantitative microscopy systems.

Although we have not been able to reliably demonstrate the use of this anti-PCNA/cyclin monoclonal antibody in formalin-fixed, paraffin-embedded material despite attempts to unmask the antigen using Pronase, tryp-

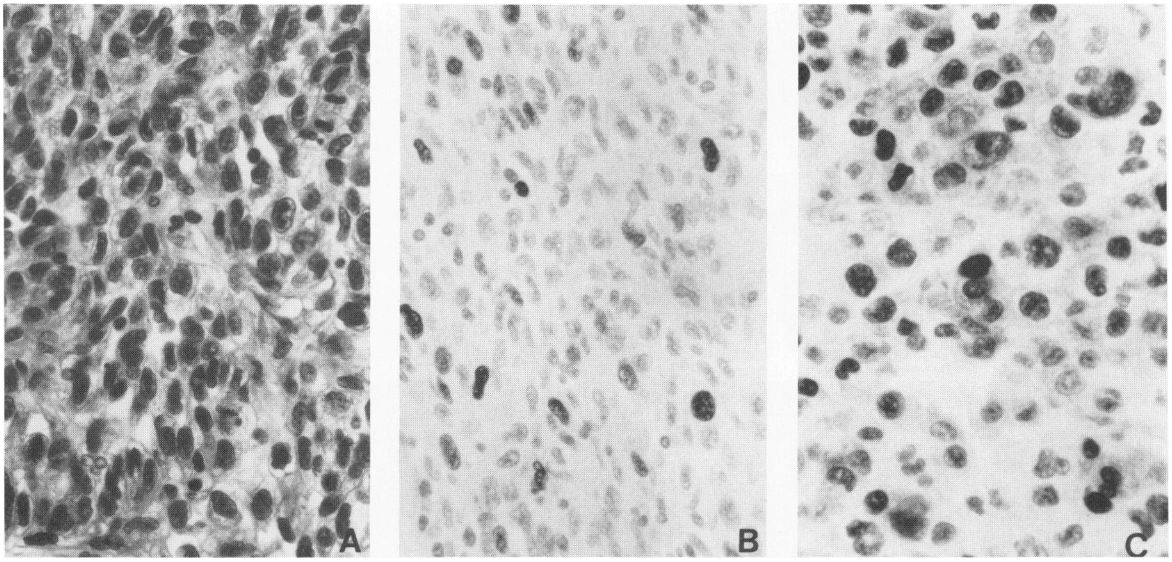

Figure 3. *Methacarn-fixed, deparaffinized sections of a metastatic, poorly differentiated malignant melanoma* **A:** *H & E-stained section.* **B, C:** *anti-PCNA/cyclin immunocytochemistry, demonstrating low* **(B)** *and high* **(C)** *proliferative indices in different areas of the same tumor. Original magnifications ×320.*

sin, and other enzymes (data not shown), this reagent joins the growing list of many diagnostically useful antibodies, such as those to the intermediate filament proteins, which are used preferentially in alcohol-based fixatives such as Carnoy's or methacarn.[21] In the one previously published study of PCNA/cyclin immunolocalization in human tumor sections, rather than the more readily available monoclonal antibody reported here, Robbins et al used purified human antisera derived from lupus patients.[22] In contrast to our studies employing the mouse monoclonal antibody 19A2, in the majority of the tumor specimens of Robbins et al, less than 10% of the nuclei were PCNA/cyclin-positive; in rare cases 20 to 50% of the nuclei were noted to be positive. This significant difference between our results and those reported by Robbins et al may be attributable to our use of an alcohol-based, rather than an aldehyde-based, fixative. It is also possible, however, that the polyclonal human antisera and the mouse monoclonal antibody differ in their tissue specificity. Nonetheless, the study of Robbins et al suggests that at least some epitopes of PCNA/cyclin can survive formalin fixation, processing, and paraffin embedding. It may thus be possible to develop additional anti-PCNA/cyclin monoclonal antibodies that can be employed on formalin-fixed, paraffin-embedded material, which would further extend the applicability of the studies reported herein.

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